

Laboratory Evaluation of Anemia

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The laboratory evaluation of anemia begins with a complete blood count and reticulocyte count. The anemia is then categorized as microcytic, macrocytic or normocytic, with or without reticulocytosis. Examination of the peripheral smear and a small number of specific tests confirm the diagnosis. The serum iron level, total iron-binding capacity, serum ferritin level and hemoglobin electrophoresis generally separate the microcytic anemias. The erythrocyte size-distribution width may be particularly helpful in distinguishing iron deficiency from thalassemia minor. Significant changes have occurred in the laboratory evaluation of macrocytic anemia, and a new syndrome of nitrous oxide-induced megaloblastosis and neurologic dysfunction has been recognized. A suggested approach to the hemolytic anemias includes using the micro-Coombs' test and ektacytometry. Finally, a number of causes have been identified for normocytic anemia without reticulocytosis, including normocytic megaloblastic anemia and the acquired immunodeficiency syndrome.

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The clinical evaluation of anemia is a common problem in medicine. During the past decade, advances have occurred in the understanding of the pathophysiology of several specific causes of anemia, and progress has been made in technology and the procedures used to do several common clinical tests. In this article I will review the current laboratory evaluation of anemia.

The use of electronic cell counters to do complete blood counts has become virtually universal. Most of them directly and precisely measure the mean corpuscular volume (MCV), hemoglobin concentrations and erythrocyte count, but only calculate the hematocrit, the mean corpuscular hemoglobin level and the mean corpuscular hemoglobin concentration. These calculations may not always be accurate, especially if the erythrocytes are deformed, as in sickle cell anemia. Because the hematocrit is calculated from the MCV and erythrocyte count, the artifact of plasma trapping does not occur as it does in a spun hematocrit. Plasma trapping is particularly prominent when the erythrocytes are misshapen or rigid, as in iron deficiency or sickle cell disease. One needs to be aware of this because if an initial hematocrit is done by microcentrifuge in the emergency department, a subsequent hematocrit in the central laboratory done by electronic counter may be much lower, when no true fall in hematocrit has occurred. Because the hemoglobin concentration is directly measured and the hematocrit calculated, the hemoglobin today is a more accurate and reproducible assessment of anemia.¹ A new piece of information, now frequently available, is a visual histogram of the distribution of erythrocyte sizes and a calculation of the coefficient of variation of the erythrocyte size expressed as the erythrocyte, or red blood cell, size-distribution width (RDW). Both of these are based on the electronic counting and sizing of 10^5 cells. The RDW, a measure of dispersion, corresponds to the clinical assessment of anisocytosis—not poikilocytosis—but is more sensitive than visual observation.

When iron, vitamin B₁₂ or folate become limiting, erythrocytes of abnormal size are produced, the visual histogram widens and the RDW increases. Occasionally two populations of cells may be observed in the histogram and, after therapy, one may be able to observe an increase in the normal size population and a decrease in the abnormal population. The ability to detect two populations, such as patients with sideroblastic anemia or those with transfusions, is limited by the height of the second population (must be more than 5%) and its separation by an observable trough from the main population.² The program used to calculate the RDW varies from instrument to instrument, and, in some cases, the histogram may contain information not conveyed by the RDW. In general, the RDW is increased with qualitative defects, such as lack of iron, vitamin B₁₂ or folic acid, but is normal in quantitative defects such as hypoplastic anemias.³ An increased RDW may be particularly helpful in distinguishing between iron deficiency and thalassemia minor.⁴ When the MCV is normal but the RDW is increased, early iron, folate or vitamin B₁₂ deficiency or a dimorphic anemia such as iron and folate deficiency may be looked for. The RDW, however, has not been rigorously tested in a prospective fashion nor outside of institutions with an interest in the area; its sensitivity and specificity in widespread clinical practice are not as yet established, and at present it does not replace the traditional evaluation. Anemias have been classified clinically by MCV for many years. The precise determination of MCV by the current machines makes the limited differential diagnosis of microcytic and macrocytic indices a useful approach.

Microcytic Anemias

The causes of an MCV of less than 80 cu microns are shown in Table 1.

In the vast majority of cases, microcytic indices are either iron deficiency or thalassemia. They usually can be distin-

ABBREVIATIONS USED IN TEXT

AIDS = acquired immunodeficiency syndrome
 ARC = AIDS-related complex
 DIC = disseminated intravascular coagulation
 EDTA = ethylenediaminetetraacetic acid
 FEP = free erythrocyte protoporphyrin
 G6PD = glucose-6-phosphate dehydrogenase
 Hb = hemoglobin
 Ig = immunoglobulin
 LDH = lactic dehydrogenase
 MCV = mean corpuscular volume
 RDW = red blood cell size-distribution width
 TIBC = total iron-binding capacity

guished easily by looking at the MCV, erythrocyte count, hemoglobin level and RDW. In iron deficiency, the progression of laboratory abnormalities is a loss of stainable bone marrow iron, a decrease in the serum ferritin level, a decrease in the serum iron content and an increase in total iron-binding capacity, anemia, a decrease in the MCV, increasing poikilocytosis and hypochromia. Generally, the MCV does not fall below 80 cu microns until the hemoglobin level is less than 10 grams per dl. The erythrocyte count decreases and the RDW increases early. The fall in the erythrocyte count is less than the fall in hemoglobin, and it is unusual to find an erythrocyte count below 2.5 million per μ l in a case of pure iron deficiency anemia. In thalassemia minor, although there is variation in cell shape and the cells are small, the variation in size is no more than normal and the RDW is normal. The body attempts to compensate for the anemia with an increase in erythrocytes. Thus, in a patient with microcytic anemia, if the erythrocyte count is normal or increased, thalassemia is the most likely diagnosis. If the hemoglobin level is greater than 10 grams per dl but the MCV is less than 75 cu microns, thalassemia is also more likely. Clinicians have used two formulas in an attempt to quantitate these relationships: (1) the Mentzer index (MCV/erythrocyte count) that, if less than 13, favors thalassemia minor and, if greater than 13, favors iron deficiency; and (2) the discriminant function (DF') of England and Fraser (DF' = MCV - [hemoglobin \times 5] - erythrocyte count - 3.4) where a negative number indicates thalassemia and a positive number iron deficiency. When the two formulas were compared with the RDW, however, the RDW proved to be much more accurate in correctly distinguishing thalassemia minor from iron deficiency.⁴ At present, even the RDW does not replace a traditional evaluation, and the diagnosis must be confirmed.

A number of laboratory techniques exist to evaluate iron stores. To be diagnostic of iron deficiency, one needs to have an iron value of less than 60 μ g per dl and a total iron-binding capacity (TIBC) of greater than 350 μ g per dl. If the TIBC is reduced, one cannot make a diagnosis of iron deficiency—no matter how low the saturation—although iron deficiency can be excluded if the saturation is more than 20% to 25%. A serum ferritin level of less than 20 ng per ml in a male patient or 10 ng per ml in a female patient is virtually diagnostic of iron deficiency.⁵ In cases of chronic infection, inflammatory disorders or renal failure, a serum ferritin level of less than 50 ng per ml is very suggestive of iron deficiency. In the final step of heme synthesis, iron is inserted into protoporphyrin by the enzyme ferrochelatase to form heme. An increase in free erythrocyte protoporphyrin (FEP) content occurs when iron is not available (such as in patients with iron deficiency or anemia of chronic disease) or the ferrochelatase is dysfunc-

TABLE 1.—*Causes of Microcytic Anemias*

Iron deficiency
Thalassemia minor
Anemia of chronic disease
Lead poisoning
Congenital sideroblastic anemia
β -Thalassemia intermedia and major
Hemoglobin H or E disease

tional (as in patients with lead poisoning). An FEP level (normal 15 to 85 μ g per dl erythrocytes) is increased in patients with iron deficiency (FEP > 100 μ g per dl erythrocytes), anemia of chronic disease and idiopathic refractory sideroblastic anemia, and usually substantially increased in those with lead poisoning (> 250 μ g per dl erythrocytes).⁶ The FEP value is usually normal in patients with thalassemia⁷ and may be used to differentiate thalassemia minor from the other causes of a microcytic anemia. A bone marrow aspirate for iron stain remains the "gold standard" for demonstrating iron deficiency anemia in difficult cases. To avoid a false-negative iron stain of the aspirate, one must obviously be certain that an adequate marrow particle has been obtained.⁸ Except in cases of severe iron deficiency anemia (hemoglobin less than 6 to 8 grams per dl), an observable reticulocyte response to iron therapy is unlikely (more precise automated reticulocyte counts are being developed that may permit detection of small increments of response). An increase in the hemoglobin content of 1 gram per dl per week and full correction of the anemia can be expected in two months, however. The diagnosis of iron deficiency mandates a search for the source of blood loss.

If thalassemia is suspected, a hemoglobin electrophoresis should be done and quantitative hemoglobin (Hb) A₂ and F levels measured. In most laboratories, a simple hemoglobin electrophoresis alone is not sufficiently accurate to measure the slight increases in Hb A₂ above the normal characteristic of β -thalassemia minor, but it will detect Hb H, E and other variant hemoglobin disorders that could present as microcytic anemia. With the decreased production of β -globin chains characteristic of β -thalassemia, the normal amounts of α -chains combine with the δ - and γ -globin to form increased amounts of Hb A₂ and F. Hb A₂ will be increased to 4% to 6% (normal < 2%). If β -thalassemia minor is complicated by concomitant iron deficiency, Hb A₂ levels may not be elevated. They will also not be increased in $\delta\beta$ -thalassemia where both genes are deleted. Hb F levels will be increased in many patients with β -thalassemia minor and all patients with $\delta\beta$ -thalassemia. If in a patient with hypochromic anemia, iron and Hb A₂ levels are normal, one is probably dealing with α -thalassemia.

In α -thalassemia, the excess β -chains have no α alternative to pair with and form β_4 -tetramers (Hb H). For the first few months of life before the γ to β switch takes place, γ_4 -tetramers (Hb Bart's) are formed in the α -thalassemias. Newborns missing one α -gene (silent carrier), two α -genes (thalassemia minor), three α -genes (Hb H disease) and four α -genes (hydrops fetalis, stillborn) can be detected and separated by their clinical features, the MCV and by the amount of Hb Bart's present.⁹ After the γ to β switch, the β_4 -tetramers (Hb H), which are much less soluble than the γ -tetramers, can only be detected in patients missing three genes (Hb H disease). In summary, if the iron studies are normal and the

hemoglobin electrophoresis is normal, the cause of a microcytic anemia is presumed to be α -thalassemia. Confirmation requires hemoglobin electrophoresis of newborn family members or restriction enzyme analysis of DNA.

The gene frequency for Hb E exceeds 10% in patients from some parts of Southeast Asia. With the recent influx of Asian immigrants to the United States, an increasing number of patients with Hb E are being seen. The substitution of lysine for glutamic acid at position 26 of the β -chain results in decreased production of β -chain due to abnormal messenger RNA splicing. Thus Hb E heterozygotes resemble patients who have β -thalassemia minor with microcytosis but without anemia.¹⁰ Homozygotes still have minimal anemia or hemolysis but with more prominent target cells and more severe microcytosis. $\beta^E\beta^0$ -thalassemia is one of the β -thalassemia intermedia syndromes. Hb E is easily detected on routine hemoglobin electrophoresis.

A hemoglobin level of less than 9 grams per dl in a patient with microcytic anemia and normal iron studies suggests Hb H disease, β -thalassemia major ($\beta^0\beta^0$) or β -thalassemia intermedia. All of these will be detected on the hemoglobin electrophoresis. Patients with β -thalassemia major (Cooley's anemia) have a severe anemia starting in infancy with massive hepatosplenomegaly, bone marrow expansion, abnormal facies and growth retardation unless maintained on a regular transfusion program. Their Hb F level is greatly increased, at times more than 90%. The β -thalassemia intermedia syndromes are characterized by a hemoglobin level of less than 9 grams per dl, but without the transfusion requirement. There are several causes including a genetic defect with some β -chain production (β^+ as in $\beta^0\beta^+$ or $\beta^+\beta^+$) or coexistent α -thalassemia with $\alpha^0\beta^0$ (the reduction of α -chain production limits the formation of unpaired α -chains that participate in the membrane damage and intramedullary destruction of erythrocytes in the pathophysiology of the anemia). Hb H disease ($-\alpha/-$) also presents with a hemoglobin level of less than 9 grams per dl, but without the long-term transfusion requirement of patients with β -thalassemia major. Like β -thalassemia intermedia and major, there is pronounced anisocytosis and poikilocytosis in Hb H disease, with nucleated erythrocytes and usually splenomegaly, features not seen in thalassemia minor. Interestingly, Hb H disease can have exacerbations of the hemolytic process induced by oxidant drugs and infections just as in glucose-6-phosphate dehydrogenase (G6PD) deficiency and the unstable hemoglobins, which will be discussed below.

Long-standing anemia of chronic disease occasionally becomes mildly microcytic, but the MCV rarely falls below 78 cu microns. In these cases, the iron level and total iron-binding capacity will both be very low, and the serum ferritin level will not be less than 100 ng per ml (normal range, 12 to 300 ng per ml, mean 23 to 34 ng per ml for menstruating women and 92 to 94 ng per ml for men).⁸ Iron deficiency and anemia of chronic disease may, of course, occur together, as in patients with inflammatory bowel disease and those with the rheumatic diseases treated with aspirin and nonsteroidal anti-inflammatory drugs. The iron saturation (iron/TIBC) may be quite low in these conditions even without iron deficiency. If, however, the saturation is greater than 30%, then iron deficiency is excluded. Because ferritin is an acute-phase reactant and serum levels tend to be elevated in inflammatory states, a serum ferritin value of less than 50 ng per ml in one of these disorders is very suggestive of iron deficiency.⁵ When

TABLE 2.—Causes of a Mean Corpuscular Volume of Greater Than 100 Cubic Microns

Megaloblastic anemia (B_{12} or folate deficiency)
Alcoholism
Liver disease
Reticulocytosis
Chemotherapy
Myelodysplastic syndromes
Multiple myeloma
Spurious
Idiopathic
Sideroblastic anemia
Hypothyroidism

ferritin levels are borderline (50 ng per ml), one may need to obtain a bone marrow aspirate for iron stain to settle the question.

Lead poisoning is a relatively uncommon cause of microcytic anemia, except in certain population groups, such as children living in an area where lead paint is prevalent or industrial workers with occupational exposure. Coarse basophilic stippling of erythrocytes is seen in lead poisoning, thalassemia and congenital erythrocyte pyrimidine 5'-nucleotidase deficiency. One needs to remember that ethylenediaminetetraacetic acid (EDTA) can abolish the stippling, so a blood smear examination should be made on a smear prepared from a finger-stick specimen of fresh blood from the tip of a needle. This practice applies not only to the search for basophilic stippling, but to evaluating peripheral blood smears in general because several artifacts can be induced by incubating blood in anticoagulated test tubes for even a few minutes to an hour. As discussed earlier, an increase in FEP levels occurs in lead poisoning and pyrimidine 5'-nucleotidase deficiency but not in thalassemia. Suspected lead poisoning should be confirmed by whole blood lead concentrations, increased urinary δ -aminolevulinic acid and coproporphyrin levels or a 24-hour urinary lead excretion after a provocative injection of EDTA.

Sideroblastic anemia is sometimes listed as a cause of microcytic anemia because of the presence of two cell populations—one microcytic—but, in fact, the MCV is always normal or slightly macrocytic,¹¹ with the exception of the congenital sideroblastic anemias. Congenital sideroblastic anemias are X-linked recessive, so primarily only males are affected. Although congenital, the onset may be delayed until adulthood. They are distinguished from acquired sideroblastic anemia by the low MCV, a uniform rather than dimorphic population of microcytic cells peripherally and the absence of dysplastic changes in marrow. This rare disorder is suggested if a microcytic anemia is found with a very high iron saturation and serum ferritin level unless the patient had thalassemia and had received many transfusions or had been mistakenly treated with iron supplements.

Macrocytic Anemias

Similar to the finding of microcytosis, the finding of an MCV of more than 100 cu microns is quite helpful because of its limited differential diagnosis (Table 2).¹² An MCV of more than 120 cu microns almost always indicates megaloblastic anemia, and, in fact, most of the time the other causes on the list will lead to an MCV between 100 and 110 cu microns. The first step is to carefully examine the peripheral smear. The finding of hypersegmented polymorphonuclear

TABLE 3.—*Causes of Falsely Low and Normal B₁₂ Levels*

Inaccurate B ₁₂ Levels	Causes
Falsely low	Folate deficiency Multiple myeloma Waldenstrom's macroglobulinemia Recent nuclear scan Third trimester of pregnancy Transcobalamin I deficiency Advanced age (see text)
Falsely normal . .	Increase in transcobalamin I and III levels Chronic myelogenous leukemia or polycythemia vera Severe liver disease

TABLE 4.—*Limitations of Erythrocyte Folate Measurements*

Inaccurate Erythrocyte Folate Levels	Causes
Falsely low	Vitamin B ₁₂ deficiency
Falsely normal	Transfusion Early folate deficiency

leukocytes in this setting means megaloblastic anemia—either vitamin B₁₂ or folate deficiency. Hypersegmentation is defined as a mean neutrophilic lobe count of greater than 3.2. Clinically, the finding of one 7-lobed neutrophil, two 6-lobed neutrophils or three 5-lobed neutrophils strongly suggests megaloblastic anemia. Other rare causes of hypersegmentation include congenital hypersegmentation and renal failure. Oval macrocytes on the peripheral smear are another sign of megaloblastosis; they are more sensitive but somewhat less specific than neutrophil hypersegmentation in predicting marrow megaloblastic changes.

In most cases, the finding of macrocytosis should prompt a determination of vitamin B₁₂ and folate levels. A low vitamin B₁₂ or folate level, however, in the absence of oval macrocytes or at least borderline hypersegmentation on a carefully examined peripheral smear should be regarded as possibly spurious, or not the primary cause of the macrocytic anemia. The old microbiologic determination of B₁₂ levels has been replaced with radioimmunoassays, and many of the methodologic problems that plagued the early radioimmunoassays have been eliminated. There remains, however, a large number of patients with a low vitamin B₁₂ level but normal bone marrow and a negative Schilling test. Some of these patients have subtle abnormalities of vitamin B₁₂ absorption due to preclinical pernicious anemia or impaired gastric function that can be elicited by tests wherein the vitamin B₁₂ is given bound to protein such as egg yolk.¹³ Many of these patients have been found to have abnormal deoxyuridine suppression.¹⁴ This can be tested only at research centers at this time and involves culturing bone marrow cells. Normally, deoxyuridine suppresses thymidine incorporation in cultured marrow cells. In the presence of vitamin B₁₂ or folate deficiency, reduced deoxyuridine suppression is corrected by the *in vitro* addition of the missing factor. This appears to be the best test for discriminating between vitamin B₁₂ and folate deficiency. Prospective studies are now under way to examine the clinical significance of a low B₁₂ level, abnormal deoxyuridine suppression, a negative Schilling test and no megaloblastic changes in the marrow. The causes of falsely low and falsely normal vitamin B₁₂ levels are given in Table 3.

Tests of serum folate levels have been replaced in many laboratories by those of erythrocyte folate levels because the

amount of serum folate is a poor reflection of tissue folate stores. Particularly problematic was the rapid correction of serum folate after one meal in hospital while tissue stores of folate remained depleted. Erythrocyte folate levels are still subject to falsely low and falsely normal results, as seen in Table 4. One of the chief drawbacks of measuring the erythrocyte folate level is that in up to half of patients with pure vitamin B₁₂ deficiency, the erythrocyte folate level will be falsely low. Conversely, as many as half of patients with folate deficiency have a low serum B₁₂ level that corrects with folate repletion alone. While some patients may be truly depleted of both vitamins, in most cases this does not appear to be so. Thus, a clinician is often faced with a patient with a megaloblastic anemia and low B₁₂ and erythrocyte folate levels.

In a patient with severe megaloblastic anemia, the safest approach would be to determine the vitamin B₁₂ and folate levels and immediately replace both vitamin B₁₂ (100 µg given intramuscularly weekly) and folate (1 mg by mouth or parenterally, then 1 mg a day); a Schilling test should be done one to four weeks later. In milder cases, one can give a trial of vitamin B₁₂ alone (1 µg per day) and observe for a brisk reticulocyte response four to ten days later. Iron stores are usually increased in megaloblastic anemia; in the absence of abundant iron, the reticulocyte response may be blunted. One could theoretically do the same thing with a very low dose of folate, such as 50 µg orally, which would correct folate but not vitamin B₁₂ deficiency. (But remember that large doses of folate will correct the hematologic abnormalities of vitamin B₁₂ deficiency but still permit the neurologic damage to progress.) Another approach to separating the two deficiencies takes advantage of the fact that the conversion of methylmalonic acid to succinyl-CoA in propionic acid metabolism is vitamin B₁₂ but not folate-dependent. Vitamin B₁₂ deficiency, but not folate deficiency, results in increased levels of methylmalonic acid in the urine. Conversely, folate but not vitamin B₁₂ participates in the conversion of formiminoglutamic acid to glutamic acid in the histidine pathway. Although urinary excretion of formiminoglutamic acid is increased in patients with folate deficiency, it may also be increased in those with vitamin B₁₂ deficiency, and this lack of specificity limits its clinical usefulness. As discussed earlier, the deoxyuridine suppression test appears to be the most sensitive indicator of early megaloblastosis and can identify the specific deficiency involved, but at this time it is only available in research centers.

If there is any doubt as to the cause of a case of macrocytic anemia, a bone marrow aspirate and biopsy should be done. In classic megaloblastic anemia, one will see increased cellularity in the biopsy specimen. The aspirate will show erythroid hyperplasia, delayed nuclear maturation relative to the hemoglobinization of the cytoplasm seen best in the basophilic, polychromatophilic and orthochromatic erythroblasts, increased cytoplasm and a characteristic megaloblastic look to the chromatin of the nucleus ("tapioca pudding"). The characteristic myeloid forms are the giant metamyelocyte and band. The myelodysplastic syndromes may also show increased marrow cellularity with megaloblastic features, but one will generally also see dysplastic features like uninuclear megakaryocytes or megakaryocytes with multiple separated nuclei, increased myeloblasts, ringed sideroblasts or more impressive erythroblast multinuclearity and nuclear fragmentation than in megaloblastic anemia.

Continuous exposure to nitrous oxide (more than six hours) can also induce megaloblastic changes in the marrow and a positive deoxyuridine suppression test with normal serum vitamin B₁₂ levels, probably by interfering with the cobalt atom in vitamin B₁₂.¹⁵ Interestingly, a diffuse polyneuropathy more sensory than motor and myelopathy affecting corticospinal tracts and posterior columns similar to subacute combined degeneration in vitamin B₁₂ deficiency may occur from occupational or recreational exposure to nitrous oxide.¹⁶ A recent report by Schilling¹⁷ suggests that nitrous oxide anesthesia may cause significant neurologic dysfunction in persons with unrecognized deficiency of vitamin B₁₂ or folate.

Once vitamin B₁₂ or folate deficiency is diagnosed, one must identify the cause. The causes of vitamin B₁₂ deficiency are reviewed in Table 5. Although anti-intrinsic factor antibody is found in only about 50% to 60% of patients with pernicious anemia, it is very specific for this disorder and therefore useful because it makes it unnecessary to do a Schilling test. Antiparietal cell antibodies are more sensitive but much less specific and therefore not helpful. Achlorhydria is also a nonspecific finding, occurring in causes of vitamin B₁₂ deficiency other than pernicious anemia and in some normal elderly patients. If one finds gastric acid, however, pernicious anemia is excluded. In summary, unless one can detect anti-intrinsic factor antibodies, the finding of a low vitamin B₁₂ level should be followed up with a Schilling test. If phase 1 is abnormal (radiolabeled vitamin B₁₂ alone) and phase 2 is normal (radiolabeled vitamin B₁₂ plus intrinsic factor) and the patient has not had a total gastrectomy, then the diagnosis is pernicious anemia. If phases 1 and 2 are abnormal, then there is small bowel disease, fish tapeworm or bacterial over-

growth. If bacterial overgrowth is suspected, then phase 2 can be repeated after two weeks of tetracycline therapy (the so-called phase 3 Schilling). On occasion, a patient with pancreatic insufficiency will fail to digest gastric R proteins that bind to the vitamin B₁₂, making it unavailable to intrinsic factor and giving a positive Schilling test.¹⁸ If the Schilling test is negative but the vitamin B₁₂ level is low and the marrow megaloblastic, then one of the more sensitive Schilling tests can be done.¹³ The double isotope Schilling test, in which phases 1 and 2 are done simultaneously using different isotopes to save time, is not recommended at present because of technical problems.¹⁹ The addition of a plasma as well as urinary determination of radioactivity appears to be a useful refinement of the traditional test to avoid the errors of incomplete urine collection. If one is aware of the potential errors in the Schilling test,¹² it remains the gold standard for the diagnosis of pernicious anemia. Patients with a new diagnosis of pernicious anemia should have a thyroid-stimulating hormone level measured because of the high incidence of Hashimoto's thyroiditis and the often cryptic presentation of hypothyroidism.

The follow-up evaluation of low folate but normal B₁₂ levels should include a review of the list of causes of low folate levels (Table 6). Unless an obvious dietary cause is found, one should evaluate small bowel function (such as with a D-xylose test, fecal fat or upper gastrointestinal tract x-ray films and small bowel follow-through).

Patients with the nonmegaloblastic macrocytic anemias almost always have an MCV of less than 120 cu microns and usually less than 110 cu microns. Alcohol ingestion (six beers or two to three drinks per day) is probably the commonest cause of mild macrocytosis.²⁰ Liver disease can also cause mild macrocytosis. Chemotherapy commonly induces a macrocytic anemia, often with some dysplastic peripheral blood and bone marrow features. Severe hypothyroidism may cause macrocytic anemia, but this is uncommon unless there is coexistent B₁₂ deficiency (Hashimoto's thyroiditis and pernicious anemia). The anemia of hypothyroidism may be normocytic or microcytic (if there is iron deficiency from heavy menstrual bleeding related to the hypothyroidism). Because the MCV of a reticulocyte is almost 160 cu microns, pronounced reticulocytosis will increase the MCV, but never to more than 120 cu microns. Furthermore, polychromasia should be obvious on a peripheral smear and easily confirmed with a reticulocyte count. Cold agglutinins and multiple myeloma can cause a falsely elevated MCV because the erythrocytes stick together and the electronic counter treats a clump as one cell. One should see agglutination on a peripheral smear in these cases, and the RDW will be increased. Spurious macrocytosis can be caused by hyperglycemia and hypernatremia because the erythrocytes are placed in isotonic medium before counting and swell.

Aplastic anemia and the myelodysplastic syndromes are commonly associated with mild macrocytosis. Features on the peripheral smear that suggest myelodysplasia include giant platelets or platelets with abnormal clumping of or decreased quantities of granules, hypogranular polymorphonuclear leukocytes or particularly bilobed neutrophils (pseudo-Pelger Huet cells). The bone marrow features of myelodysplasia were discussed earlier in the differential of megaloblastic marrow.²¹ A bone marrow diagnosis is essential for accurately determining a prognosis with regard to the risk for transformation to acute leukemia.

TABLE 5.—Causes of True Vitamin B₁₂ Deficiency

Pernicious anemia
Gastrectomy or severe gastritis
Pancreatic insufficiency
Disease or resection of the terminal ileum
Bacterial overgrowth of small bowel
<i>Diphyllobothrium latum</i>
Transcobalamin II deficiency
Continuous administration of nitrous oxide
Strict vegetarianism

TABLE 6.—Causes of True Folate Deficiency

Dietary deficiency, especially in association with alcoholism
Pregnancy
Chronic hemolytic anemia, such as sickle cell anemia
Chronic desquamative skin diseases, such as psoriasis
Renal failure with hemodialysis or peritoneal dialysis
Drugs
Phenytoin
Sulfamethoxazole-trimethoprim
Methotrexate
Alcohol
Oral contraceptives
Severe small bowel disease
Crohn's disease
Lymphoma or leukemia infiltration
Tropical and nontropical sprue
Scleroderma
Diabetes mellitus
Amyloidosis

Normocytic Anemia With Reticulocytosis

There are three causes of normocytic anemia with an increased number of reticulocytes: bleeding, recent correction of a production defect and hemolysis. In the absence of obvious external or occult gastrointestinal blood loss, one should consider occult retroperitoneal, pelvic or deep muscle bleeding. Anemia with an increased reticulocyte count may occur following repletion of B₁₂ or folate (note that the neutrophil hypersegmentation will persist for 7 to 14 days)²² but not usually with iron replacement. This may also occur with removal of a marrow suppressant like alcohol or with the treatment of infection. The third cause of a normocytic anemia with an increased reticulocyte count is hemolysis. Hemolysis is suggested by elevated bilirubin and lactic dehydrogenase (LDH) levels, a peripheral smear showing schistocytes or spherocytes, or dark urine. While an elevated LDH level is nonspecific, a normal LDH value is against ongoing hemolysis and an extremely high LDH favors it. The bilirubin is roughly proportional to the erythrocyte mass so that a bilirubin of 1.0 mg per dl with a hemoglobin of 10 grams per dl and an increased reticulocyte count is suggestive of hemolysis.²³

If the number of reticulocytes is reported as a crude percentage, then it should be corrected for the degree of anemia by multiplying the observed reticulocyte count by the observed hematocrit concentration divided by 45 to achieve a more meaningful assessment of marrow production. Normally, reticulocytes are recognizable by a supravital stain in the peripheral blood for one day after they are released from the marrow. With increasing degrees of anemia, the reticulocytes are released earlier from the marrow and therefore are present longer in the peripheral blood. Thus at a hematocrit of 35%, the reticulocyte lifespan in the peripheral blood is 1.5 days; at a hematocrit of 25% it is 2 days, and at a hematocrit of 15% it is 2.5 days.²⁴ To accurately assess the daily reticulocyte production, a second correction should be made for this "shift" phenomenon. The above reticulocyte "index" or absolute reticulocyte percentage is divided by 2 for a moderate anemia (with a hematocrit of around 25%) and divided by 3 for a severe anemia (with a hematocrit of around 10%). In response to acute bleeding or hemolysis, a normal bone marrow should increase its daily production to 2% reticulocytes at a hematocrit of 35%, and to 3% with severe anemia (hematocrit of 10%).

In the further evaluation of suspected hemolysis, it is useful to determine whether erythrocytes are being destroyed intravascularly or extravascularly (in the spleen) because only a limited number (Table 7) of disorders causes intravascular hemolysis and because intravascular hemolysis may result in acute renal failure and disseminated intravascular coagulation (DIC). The intravascular lysis of even a few erythrocytes will turn the plasma red or brown. The hemoglobin is picked up by haptoglobin until the haptoglobin is saturated, at which time free hemoglobin is filtered at the glomerulus resulting in hemoglobinuria. The urine will be red or brown and dipstick-positive for blood, but without erythrocytes on microscopic examination. Although the urine in cases of myoglobinuria will appear the same, the plasma is clear because the smaller myoglobin molecule is so rapidly cleared. Some of the hemoglobin that is filtered by the glomerulus will be taken up by renal tubular epithelial cells that slough several days later, leading to a positive urine hemosiderin test. Increased LDH levels, reduced haptoglobin and increased indirect bilirubin

TABLE 7.—*Causes of Intravascular Hemolysis*

Glucose-6-phosphate dehydrogenase deficiency
Microangiopathic hemolytic anemia
ABO-incompatible blood transfusion
Cold antibody autoimmune hemolytic anemia (cold agglutinin disease)
Warm antibody autoimmune hemolytic anemia
Paroxysmal cold hemoglobinuria
Paroxysmal nocturnal hemoglobinuria
Clostridial sepsis
Delayed hemolytic transfusion reaction

TABLE 8.—*Causes of Microangiopathic Hemolytic Anemia*

Disseminated intravascular coagulation
Thrombotic thrombocytopenic purpura
Malignant hypertension
Eclampsia
Vasculitis
Metastatic cancer
Extreme turbulence due to major abnormalities within the heart or great vessels
Sepsis

occur with both intravascular and extravascular hemolysis. Therefore, the first steps in evaluating suspected hemolysis are to visually inspect the plasma in a spun specimen, to inspect the urine and examine it under the microscope for erythrocytes and to test it with a dipstick for blood.

If there is evidence of intravascular hemolysis, the next steps are to examine the smear for evidence of microangiopathic changes and to order a Coombs' test and a Heinz body preparation. At times, the microangiopathic changes may be relatively subtle; if there is one or two schistocytes, fragmented cells or helmet cells per high-power field, then one needs to consider the differential diagnosis of the microangiopathic syndromes (Table 8). If the platelet count is low or falling, one needs to strongly consider DIC, thrombotic thrombocytopenic purpura or the hemolytic uremic syndrome. Prolongation of the thrombin time, prothrombin time and partial thromboplastin time, reduction of the fibrinogen level, elevation of the fibrin-split products and a positive fibrin monomer are seen in patients with DIC, but the results of these tests are relatively normal in thrombotic thrombocytopenic purpura and the hemolytic uremic syndrome. Fever, a fluctuating neurologic status and only mildly abnormal blood urea nitrogen and creatinine levels in an adult suggest thrombotic thrombocytopenic purpura. Acute oliguric renal failure with a relatively normal neurologic status in a child suggests the hemolytic uremic syndrome.

While warm autoimmune hemolytic anemia only rarely causes intravascular hemolysis, it is easily discovered by a positive direct Coombs' test, reflecting immunoglobulin (Ig) G or C3 on the surface of the erythrocytes. If one finds only C3 on the surface of the erythrocytes, then the diagnosis is most likely cold agglutinin disease. The IgM is bound to the erythrocytes in cooler areas of the body and fixes C3, but upon rewarming, the IgM diffuses off, leaving C3 alone when the Coombs' test is done.

If there is evidence of intravascular hemolysis but the peripheral smear is normal and the Coombs' test is negative, one should suspect G6PD deficiency. A Heinz body preparation may show the oxidized and precipitated hemoglobin

during and briefly after an episode of acute G6PD hemolysis. In the African or B-type of G6PD deficiency, which occurs in 12% of American blacks, the enzyme is unstable and has a shorter than normal half-life so that the older erythrocytes have the lowest levels and are susceptible to hemolysis with infection, acidosis or oxidant drugs. In these patients, the hemoglobin level will drop suddenly from normal to about 8 to 9 grams per dl and then stop or even rise again. Because all of the older cells have been destroyed, one will have to wait six to eight weeks and then assay the G6PD. In the Mediterranean and Chinese varieties of G6PD deficiency, the defect is more profound and affects all cells so that patients' hemoglobins will not stabilize at 8 to 9 grams per dl, but may fall to extremely low levels if the oxidant stress continues. In these patients, the G6PD assay is often diagnostic even after an episode of hemolysis.

Two other causes of intravascular hemolysis are paroxysmal cold hemoglobinuria and paroxysmal nocturnal hemoglobinuria. Paroxysmal cold hemoglobinuria is due to a cold-reactive IgG that on rewarming activates the full complement sequence with brisk intravascular hemolysis. The cold IgG—the Donath-Landsteiner antibody—used to be seen in patients with congenital and tertiary syphilis, but today is usually postviral or idiopathic. One needs to specifically do a Donath-Landsteiner test because the Coombs' test may be negative. In paroxysmal nocturnal hemoglobinuria, the hemoglobin level is usually low, but may be normal. The leukocyte and platelet counts are usually reduced. Paroxysmal nocturnal hemoglobinuria should always be considered in the differential diagnosis of pancytopenia, particularly with increased reticulocytes. The leukocyte alkaline phosphatase score is low in cases of this disorder. The two best screening tests are the urine hemosiderin and the sucrose hemolysis tests. The urine hemosiderin test is almost always positive. The sucrose hemolysis may on occasion be normal, particularly after an episode of hemolysis has reduced the number of abnormal cells. A positive sucrose hemolysis test should be confirmed with the complete Ham's acid hemolysis test.

In the absence of hemoglobinemia or hemoglobinuria, the hemolysis may still have been intravascular and missed, but is probably extravascular. It is useful to know what a previous complete blood count was because almost all new cases of hemolytic anemia in adults are autoimmune hemolytic anemia, G6PD deficiency or, less commonly, microangiopathic hemolytic anemia. Evaluation begins by examining the smear. The significance of microangiopathic changes has been discussed. True spherocytes (small, darkly pigmented and without central pallor) suggest autoimmune hemolytic anemia or hereditary spherocytosis; spherocytes may also be seen with severe burns. Therefore, in a new case of hemolytic anemia, spherocytes are very suggestive of autoimmune hemolytic anemia. A Coombs' test should be done even if the erythrocytes look normal. If spherocytes are seen and the Coombs' test is negative, an osmotic fragility test (or, preferably, ektacytometry) should be done.²⁵ Ektacytometry is a new technique, not widely available, that appears to be more sensitive than simple osmotic fragility testing for hereditary spherocytosis and other defects of the erythrocyte cytoskeleton. The erythrocytes are suspended in an osmotic medium that can be varied from hypotonic to hypertonic, simultaneously subjected to a shear stress and their instantaneous deformability measured by laser diffraction. If the Coombs' and osmotic fragility tests are negative, a "micro" or

"super" Coombs' test should be done. A standard Coombs' test will detect more than 500 molecules of IgG per erythrocyte. The micro-Coombs' test will detect between 50 and 500 molecules per cell, which occurs in 2% to 5% of cases of autoimmune hemolytic anemia. The new onset of hemolytic anemia with a normal smear and a negative Coombs' test, especially in the proper population group, is very suggestive of G6PD deficiency. As noted above, 12% of black men and boys in this country are affected by this X-linked recessive disorder; 1% of black women and girls are homozygous and affected and 25% of black women and girls are carriers, of whom a fourth are subject to hemolysis by lyonization with excessive inactivation of the normal X chromosome.

If the answer is not forthcoming from the above evaluation, it is helpful to retreat to a more formal consideration of the causes of hemolysis. Traditionally, these are divided into defects within erythrocytes, in the membrane of erythrocytes and outside of erythrocytes. Within erythrocytes, one may have defects in either the hemoglobin or the enzymes. Sickle cell anemia is obvious from a smear. The thalassemias are microcytic and have been discussed above. It is worthwhile to recall that Hb H disease ($-\alpha/\alpha$) can have drug- and infection-induced exacerbations of hemolysis like G6PD deficiency. The unstable hemoglobins are uncommon and usually present as a chronic nonspherocytic hemolytic anemia from childhood. Some, however, may be mild, but patients can have oxidant-induced hemolysis and therefore the disorder appears as an acquired hemolytic anemia. Unstable hemoglobins are detected with incubation with brilliant cresyl blue, resulting in the formation of Heinz bodies.²⁶ This will also occur in patients with Hb H disease and G6PD deficiency. Other tests include hemoglobin electrophoresis and incubation with 17% isopropanol at 37°C or heating to 50°C to look for instability and precipitation.¹⁷ Of the enzyme deficiencies, G6PD is by far the most common. Pyruvate kinase is the next most common enzyme deficiency and accounts for 95% of the glycolytic pathway deficiencies. Glucose phosphate isomerase accounts for about 4% and the rest are extremely rare.²⁷ Pyrimidine 5'-nucleotidase deficiency causes basophilic stippling and a microcytic anemia.

Of the membrane defects, hereditary spherocytosis, elliptocytosis and stomatocytosis should be obvious from the peripheral smear. Major advances have occurred in our understanding of the molecular basis of these disorders, all of which appear to involve defects in the erythrocyte cytoskeleton. Although these disorders are congenital, it is not uncommon for them to be detected for the first time in adulthood. Any insult that causes the marrow to stop producing may cause an aplastic crisis with a rapid fall in the hematocrit. In addition, these patients have gallstones at an early age and are at some increased risk of splenic rupture from their splenomegaly. All three disorders will show increased autohemolysis when defibrinated sterile blood is incubated for 48 hours, although this test is rarely done today. In addition, the results of osmotic fragility testing and ektacytometry will be abnormal.

Defects outside the erythrocyte besides autoimmune hemolytic anemia and microangiopathic processes include sepsis (especially clostridial sepsis), severe hypophosphatemia (< 1 mg per dl) and Wilson's disease.

Normocytic Anemia Without Reticulocytosis

The differential diagnosis of anemia with an erythrocyte MCV of between 80 and 100 cu microns without an increase

TABLE 9.—*Causes of Normocytic Anemia Without Increased Reticulocytes*

Anemia of chronic disease
Early iron deficiency
Megaloblastic anemia with α -thalassemia or iron deficiency
Hypoadrenal states
Renal failure
Acquired immunodeficiency syndrome
Aplastic anemia
Pure red cell aplasia
Anorexia nervosa
Bone marrow infiltration
Leukemia
Lymphoma
Cancer
Granulomatous diseases
Myeloproliferative disorder

in reticulocytes is shown in Table 9. Within the past few years, it has been recognized that megaloblastic anemia may occasionally be normocytic,²⁸ particularly in populations with an increased prevalence of α -thalassemia or iron deficiency. One would still expect to find hypersegmentation of polymorphonuclear leukocytes and oval macrocytosis, and the RDW would be increased. Probably the most common causes of a decreased-production normocytic anemia are early iron deficiency and the anemia of chronic disease. While the MCV generally does not fall below 80 cu microns in iron deficiency until the hemoglobin level is less than 10 grams per dl, the RDW will increase very early in the development of iron deficiency. Measurement of the serum iron level, TIBC and the serum ferritin level will resolve this. The pathophysiology of the anemia of chronic disease that involves decreased release of iron from reticuloendothelial stores, shortened red cell survival and decreased response to erythropoietin occurs within hours of the onset of infection or inflammation, and one may reasonably expect the hematocrit of a sick patient in hospital having daily blood tests to fall by 1% per day. One needs to assess the blood urea nitrogen and creatinine levels because renal failure is a common cause of decreased-production anemia. Hypoadrenal states and, in particular, hypothyroidism also result in a hypoproliferative anemia. Due to its often subtle presentation, particularly in the elderly, one should have a low threshold of suspicion for checking the thyroid-stimulating hormone levels. Hypersplenism may cause a normocytic anemia with or without mild to moderate reticulocytosis. Generally one will also have moderate thrombocytopenia and leukopenia. The spleen need not be palpably enlarged. The bone marrow is generally hyperplastic and nonspecific unless also involved by the process causing the hypersplenism.

Multiple myeloma may present as a normocytic or macrocytic anemia and may be screened for with a serum protein electrophoresis and urinary immunoelectrophoresis. Myelofibrosis presents a leukoerythroblastic smear with nucleated and teardrop-shaped erythrocytes, an increase in the leukocyte count or the presence of early myeloid forms and giant platelets. Similar changes, but usually of a lesser degree, may be found with metastatic cancer, leukemia, lymphoma or granulomatous disease involving the marrow. There are, however, a number of benign disorders that can mimic these features²⁹ so that a bone marrow aspirate, biopsy and specimens for culture need to be obtained. Similar to the leukoery-

throblastic disorders, the diagnosis of pure red cell aplasia or aplastic anemia requires examining the bone marrow. Thus, unless one finds evidence of iron deficiency, renal failure or hypothyroidism, a bone marrow examination is necessary.

Many patients with the acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) will have anemia or pancytopenia in the course of their illness. The peripheral smear is usually unrevealing. The findings of a bone marrow examination in patients with AIDS or ARC are usually nonspecific with increased cellularity, an increase in plasma cells and eosinophils and often some fibrosis. Mycobacterial infections in AIDS patients may occur without granulomas, emphasizing the importance of specific stains for acid-fast bacilli and culturing the marrow for bacteria, fungus and typical and atypical mycobacteria.

Conclusion

The clinical evaluation of anemia begins with the history, physical examination, complete blood count including RDW, reticulocyte count and examination of the peripheral smear. With these data, one generates a limited differential diagnosis, with one or two statistically likely possibilities and a few to several uncommon possibilities. During the past decade, the procedures used for confirming suspected vitamin B₁₂ and folate deficiencies have changed, and one needs to be aware of the causes of falsely low and falsely negative studies. The procedures for delineating the causes of microcytic and normocytic anemias with or without reticulocytosis have been refined but remain fundamentally unchanged. A systematic but focused approach to the laboratory evaluation of anemia results in expedient and precise diagnosing and avoids unnecessary testing.

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Software Review

The Western Journal of Medicine does not review all books or other material sent by publishers, although information about new books received is printed elsewhere in the journal as space permits. Prices quoted are those given by the publishers.

Hypertensive Emergencies

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Review of Software

In the past two years, a number of patient simulation programs have been written to run on microcomputers. Examples include programs by CME, Inc., associated with the University of Washington, the Scientific American Discotests, and the Williams and Wilkins Rx/Dx series. This last series of ten or so programs deal primarily with cardiology issues. They have been authored by a group of physicians from the Massachusetts General Hospital. A recent addition to this series is "Hypertension Emergencies," authored by Edward Hoffer and Octo Barnett, both of the Laboratory of Computer Science at Massachusetts General Hospital.

The program comes on one disk, with a small booklet about using the program and a form to reply for CME credit (five hours). At \$85, the price is a little steep when considered either as a computer program or home-based CME program. The program "boots" easily and is primarily menu based. One gets a choice of 18 patient simulations. Each simulation gives a brief history and physical, and then one primarily decides on pharmacological management.

The simulations are relatively short (five to ten screens), so that one doesn't get bored or engrossed in minutia. The directions are clear, and the program ran flawlessly. For a right answer chosen, the program gives a good pat on the back; if a wrong answer was chosen, there are additional loops that one goes through with screens of information and further questions. The screen fills in a jerky fashion from bottom to top, however, much like a piece of paper out of a printer, so that one has to wait for the screen to fill and stop moving before reading. The program was copyrighted in 1985, so that some of the information on the more newly released drugs is out of date.

My major criticism has to do with the direction and content of the program. Of the 18 cases, 17 dealt almost exclusively with drug management. The program sticks religiously to the stepped care approach. The program was a little more aggressive in recommending pharmacological treatment than I am (for instance recommending increased drug therapy in a middle-aged man with a blood pressure of 130/90). The patient education recommendations primarily emphasized getting the patients to take their medication each day. Only one of the simulations stressed low salt diet and exercise (but did give some good references). Many of the simulations seemed very repetitive, with a disproportionate number being black men who had very significant elevations of blood pressure that responded to diuretics and beta-blockers. Behavioral approaches and other nonpharmacologic treatments were rarely mentioned.

In conclusion, I think this program should be relabeled the "Pharmacologic Management of Hypertension." For most practicing physicians, I don't think it significantly adds to one's knowledge, unless one is not familiar with the stepped care approach and a couple of second line agents. The program might be more appropriate for medical students who are beginning to learn about decision making and drug therapy of hypertension. Overall, this is a straightforward but somewhat simple application of computer-based patient simulation. Its usefulness is limited.

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